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Effects of a novel microsporidium on the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae)

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ABSTRACT

A newly discovered microsporidium infecting the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae), provisionally placed in the genus *Canningia*, was studied to determine its impact on *O. sulcatus*. *O. sulcatus* populations from several locations were sampled and evaluated for microsporidiosis. A very low prevalence of the disease was observed in all locations surveyed (<3.0%). Laboratory studies were conducted by orally exposing both larvae and adults of *O. sulcatus* to varying concentrations of *Canningia* sp. spores. Larval bioassays at a variety of dosages (0, 10, etc.) were performed to evaluate pathogen infectivity, larval survival and growth. Adult bioassays (dosages: 0, 10, etc.) were performed to evaluate longevity, fecundity and mechanisms of vertical pathogen transmission. Larvae and adults were infected in all spore treatments. Larval growth was significantly reduced at dosages above 10 spores/larva. Adults infected at all dosages experienced high levels of mortality and fecundity was reduced to zero. Greenhouse trials were performed to determine if larvae feeding in soil acquired infections when spores were topically applied as a drench application (0, 10^5, 10^6, 10^7 spores/pot). Established larvae feeding on plant roots in pots developed infections when exposed to drench treatments of 10^6 and 10^7 spores/pot after 14–21 days. *Canningia* sp. is an acute pathogen of *O. sulcatus* infective to both larvae and adults. Topically applied spores also infected larvae feeding on roots in soilless potting media, suggesting the possibility of using this pathogen in a microbial control program.

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1. Introduction

The black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae) is a univoltine, polyphagous insect that is a severe pest of both field and container-grown ornamentals as well as small fruit crops worldwide (Moorhouse et al., 1992). *O. sulcatus* originated in northern Europe and was first recorded in North America in 1835. The ultimate instar is the primary overwintering stage. In regions where mild winters are typical, a minor proportion of the adult weevil population survives the winter. Pupation and adult eclosion occur in the spring. Adult weevils are nocturnal and cause largely cosmetic damage by notching plant leaves. *O. sulcatus* has a preovipostion period of 20–40 days, feeding on leaves while its reproductive system matures (Smith, 1932). Reproduction is by thelytokous parthenogenesis, so a single individual left unchecked can result in an infestation. Depending on the host plant fed upon by an adult, each individual can lay nearly 900 eggs (Fisher, 2006). Oviposition occurs at night with eggs deposited on the soil surface or inserted into soil crevices (Smith, 1932). Early instars feed on small roots while the later instars feed on larger roots, especially on the phloem and cambium tissues near the soil surface (LaLone and Clarke, 1981). Larval feeding can be quite severe (Moorhouse et al., 1993).

The *O. sulcatus* control program implemented by a majority of small fruit and nursery growers centers on the use of broad spectrum insecticides targeted against preovipositional adults. Infestations are particularly problematic in the ornamental nursery industry in which there is a zero tolerance for *O. sulcatus* infestations. Infested nursery stock cannot be sold and if infested plants are inadvertently shipped, growers risk refusal of the plants by the buyer and will incur the additional return shipping costs and potential loss of future sales. Many of the chemical insecticides currently available for curative applications (i.e. drenches in the late fall and early spring) do not adequately control established *O. sulcatus* larval infestations. Often chemicals applied targeting established *O. sulcatus* larval infestations are not effective against the last instar because they are difficult to apply effectively to large nursery containers (D.J.B., personal observation). Entomopathogenic nematodes are currently available and commercially used as a curative application for *O. sulcatus* control in container-grown nursery stock. Entomopathogenic nematodes are available in a number of different commercial formulations and are most often applied with agricultural sprayers or irrigation equipment (Grewal,

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2002). When applied during periods of favorable soil temperatures, nematodes are efficacious for *O. sulcatus* larval control (Bruck et al., 2005). The nursery industry is continuously looking for new effective alternatives for ridding containers of existing *O. sulcatus* infestations.

Microsporidia are obligately parasitic single-celled eukaryotes that as a group infect insects from nearly every order (Solter and Becnel, 2007). The infective stage of a microsporidium is the mature environmentally resistant spore, which must be orally ingested. Nearly all microsporidia possess a polar filament, a unique infection apparatus that is everted from the spore when infecting the host cell. Most species cause chronic infections, the effects of which can be benign to severe. Commonly seen fitness costs associated with microsporidian infection include reduced longevity and fecundity of adults; increased larval mortality and developmental time; and susceptibility to environmental stressors (Siegel et al., 1986; Bruck et al., 2001; Fuxa et al., 2005; Joudrey and Bjornson, 2007). Microsporidian infection can also have adverse affects on both egg and larval parasiotoids (Cossentine and Lewis, 1987, 1988; Orr et al., 1994; Schuld et al., 1999; Hoch et al., 2000). Relatively few of the currently described microsporidia have been studied extensively for potential use as microbial control agents, primarily due to issues such as complicated life cycles, obligatory parasitism, typically cause chronic rather than acute infections, low persistence in the field and storage difficulties (Brooks, 1988).

A new microsporidium species was recently recovered from a field population of *O. sulcatus* in May, 2003 at a commercial wholesale ornamental nursery located in McMinnville, OR. Initial taxonomic studies show it to be genetically aligned with the *Nosema/Vairimorpha* group (GenBank EU589246), but it lacks one of the major characteristics of the clade, a diplokaryotic nucleus throughout the lifecycle of at least one spore type (L. Solter, D. Bruck, M. Baker, unpublished data). Although genetically basal to the *Nosema*/*Vairimorpha* clade, this microsporidium possesses morphological characteristics (monokaryotic throughout the lifecycle and isolated from beetles) of the relatively recently described genus *Canningia* (Weiser et al., 1995; Kohlmayer et al., 2003), and is provisionally placed in this genus pending further analysis and formal description.

Microsporidia have been isolated from a handful of other closely related *Otiorhynchus* species; all of which were described as belonging to the genus *Nosema* (Hesse, 1905; Weiser, 1951; Sprague, 1977; Hostounsky and Weiser, 1981). These microsporidia were described primarily from infected adults, all of which were collected from the field in Europe and were described prior to the availability of sequence data to assist in their proper placement. Microsporidia previously isolated from otiorhynchids infected a range of host tissues (Hesse, 1905; Weiser, 1951; Sprague, 1977; Hostounsky and Weiser, 1981) and presumably were diplokaryotic (based on the requirement for the genus *Nosema*), while *Canningia* sp. is monokaryotic and infection in *O. sulcatus* larvae is limited to the gut tissues (unpublished data).

2. Materials and methods

2.1. Prevalence in the field

A survey was conducted (2005 and 2006 growing seasons) to determine the natural prevalence of *Canningia* sp. in the field. *O. sulcatus* (larvae and adults) were collected from a number of field sites (small fruit, nursery, and riparian areas) around North America (Table 1). All specimens collected from locations outside of Oregon were stored in the freezer (−20 °C) until shipped. Specimens collected in Oregon were returned to the laboratory and immediately frozen. From each location from which *O. sulcatus* were collected, up to 25 weevils were randomly selected from each collection date and examined for presence of the microsporidium. Because infections are limited to the gut, the entire gut of each weevil was removed to make a fresh tissue smear on a glass slide. Smears were examined at 400× for the presence of *Canningia sp* spores.

Table 1
Prevalence of *Canningia* sp. infection in black vine weevil field collected from various field and nursery locations

Location	Stage evaluated	*n*	Number infected	Percent infection
McMinnville, OR[a]	Larvae/adults	217	6	2.7
Oregon[b]	Larvae/adults	410	1	0.24
New York[b]	Adults	50	0	0
Ont., Canada	Adults	322	5	1.5

[a] Nursery location at which *Canningia* sp. was originally isolated.
[b] Cumulative infection of weevils pooled from various sample locations around the state.

2.2. Larval response and development

Bioassays were performed to determine larval survival and developmental responses to varying dosages of *Canningia* sp. Third instar *O. sulcatus* were obtained from a laboratory colony maintained at the USDA-ARS Horticultural Crops Research Laboratory (HCRL) (Fisher and Bruck, 2004). Spores were produced *in vivo* by infecting third to fourth instar *O. sulcatus* with 10^2 spores and harvested immediately prior (~14 days post-infection) to the initiation of the bioassays. Healthy larvae were individually presented small cubes ($1 \times 1 \times 1$ mm) of meridic diet (Fisher and Bruck, 2004) in 96-well microwell plates. Forty larvae were exposed to diet cubes treated with 0, 10^1, 10^2, 10^3, 10^4 and 10^5 microsporidium spores for 48 h. Twenty larvae that had consumed their entire diet cube were randomly selected and placed individually into 3 oz cups containing fresh meridic diet. Larvae were observed every 2–3 days and mortality recorded. Larvae that died during the course of the experiments were immediately examined for the presence of the microsporidium, as described previously. After 21 days, surviving larvae were weighed and also evaluated for microsporidian infection. An arcsine transformation of the percentage larval mortality was performed to stabilize variance (Snedecor and Cochran, 1989). Data from the bioassays (percent survival and larva weight) were analyzed using the General Linear Models Procedure (GLM) with Tukey's multiple range test used to separate means (SAS Institute, 1999).

2.3. Soil drench applications

Experiments were performed to determine if spores, topically applied to the soil surface of container-grown nursery plants, resulted in larval infection. Spores were produced *in vivo* by infecting third to fourth instar *O. sulcatus* with 10^2 spores and harvested ~14 days post-infection. Rooted cuttings of *Picea abies* 'Nidiformis' were potted into four inch pots with soilless potting media (SB40, Sun-Gro Horticulture, Bellevue, WA) typical of that used in the nursery industry. Pots were artificially infested with five healthy third and fourth instar *O. sulcatus* obtained from the HCRL colony 1 week prior to spore application. At the time that pots were infested, an additional 50 larvae from the same cohort were examined to verify the absence of the microsporidium in the larvae used in the study. No infections were observed. The experiment was arranged in a randomized complete block design with three replications, each containing nine pots, and four levels of spore treatment (0, 1×10^5, 1×10^6 and 1×10^7 spores/pot). Spores were topically applied to each pot in 75 ml of water using a graduated cylinder. Pots

were watered lightly after spores were applied. The experiment was maintained in the greenhouse at 21 °C, 16:8 (L:D) for 14 days. Four pots from each treatment were evaluated after 7 days and the remaining five pots were evaluated after 14 days. All test larvae were recovered from each pot and examined for infection as described previously. This experiment was subsequently repeated using the same methods with the following alterations. Because of a lack larval infection from all treatments after 7 days exposure; evaluations for larval infection were made after 14 and 21 days. Because of an absence of larval infection at 14 days post-treatment at 1×10^5 spores/pot in the first trial of the experiment, this treatment was eliminated in the second trial. An arcsine transformation of the percentage larval infection was performed to stabilize variance (Snedecor and Cochran, 1989). Data from the bioassays were analyzed using the General Linear Models Procedure (GLM) with Tukey's multiple range test used to separate means (SAS Institute, 1999).

2.4. Adult survival and fecundity

Multiple attempts were made to infect late instar *O. sulcatus* with low dosages (10–100 spores) of *Canningia* sp. in order to determine the effects on adult survival and fecundity, as well as on the ability to be transmitted vertically. However, even late instars receiving these very low dosages did not successfully pupate and eclose. We therefore inoculated preovipositional adults obtained from the HCRL colony with *Canningia* sp. spores presented on 5 mm strawberry 'Totem' leaf discs. Forty adults were exposed for 48 h to leaf discs treated with 0, 10^3 10^4, 10^5 and 10^6 microsporidian spores. Twenty adults that consumed the entire leaf disc were randomly selected and placed individually into 100 mm Petri plates (VWR International, West Chester, PA) containing fresh strawberry 'Totem' leaves. Adults were observed every 2–3 days for mortality and any eggs laid were collected weekly for up to 3 weeks after the onset of oviposition. Adults that did not lay eggs by 6 weeks after treatment were sacrificed and examined for infection. Adults that died during the course of the study were also examined for the presence of microsporidian spores. After the onset of oviposition, up to 10 eggs from each adult were randomly selected each week and the percentage of egg hatch determined. To determine hatch, 10 eggs were placed on damp filter paper in 50 mm snap-lock Petri plates (Becton Dickinson, Franklin Lakes, NJ) and incubated at 21 °C for 2 weeks. An arcsine transformation of the percentage adult mortality and infection was performed to stabilize variance (Snedecor and Cochran, 1989). Data from the bioassays were analyzed using the General Linear Models Procedure (GLM) with Tukey's multiple range test used to separate means (SAS Institute, 1999).

3. Results and discussion

Inoculation of third instar *O. sulcatus* larvae with 100 or more *Canningia* sp. spores resulted in a significant reduction in larval survival ($F = 10.97$; df = 5, 114; $P < 0.0001$). Surviving larvae from the microsporidian treatments also weighed significantly less ($F = 25.54$; df = 5, 55; $P < 0.0001$) than uninfected larvae 21 days post-treatment (Table 2). All larvae fed *Canningia* sp. spores developed infections. There was no significant difference in larval survival between control larvae and those larvae ingesting 10 spores (Table 2), however this experiment was terminated at 21 days. Later experiments indicated that larvae fed as few as 10 spores failed to complete pupation. The effects of infection were severe. Surviving larvae that ingested 100 or more spores were significantly smaller in size than the control larvae or those fed 10 spores. Larvae fed higher spore dosages that survived 21 days were moribund and their potential to survive in the field is low.

Table 2
Impact of *Canningia* sp. dosage on the survival, growth and fecundity of black vine weevil larvae after 21 days and adults after 6 weeks (means ± SD)

Spore dosage	Larvae[a]		Adults[a]	
	Percent Survival	Larval Weight (mg)	Percent Survival	Fecundity[b]
0	100 ± 0a	67 ± 5a	76 ± 25a	30 ± 10a
1×10^1	75 ± 22ab	59 ± 9a	—[c]	—
1×10^2	45 ± 25bc	40 ± 11b	—	—
1×10^3	15 ± 18c	21 ± 2bc	26 ± 20b	0 ± 0b
1×10^4	40 ± 25bc	17 ± 2c	17 ± 20b	0 ± 0b
1×10^5	30 ± 20c	18 ± 2c	9 ± 9b	0 ± 0b
1×10^6	—	—	0 ± 0b	0 ± 0b

[a] Means in the same column followed by the different letters are significantly different ($P < 0.05$; SAS Institute, 1999). Twenty individuals evaluated at each dose.
[b] Eggs/♀ in the initial 3 weeks of oviposition.
[c] Treatment not included.

Canningia sp. was successfully applied as a drench to the surface of pots containing plants infested with *O. sulcatus* larvae. Although microsporidia have been successfully applied experimentally against a number of foliar insects including the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) (Lewis and Lynch, 1978; Lublinkhof et al., 1979; Lublinkhof and Lewis, 1980), *Lacanobia oleracea* (Down et al., 2004), and gypsy moth, *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) (Weiser and Novotny, 1987; Jeffords et al., 1989), we are unaware of any reports of the successful application of microsporidia against a root-feeding insect. In the first trial of the drench application experiment, there was a significant day by dosage interaction on the percentage of infected *O. sulcatus* larvae after 14 days ($F = 8.88$; df = 7, 100; $P < 0.0001$; Table 3). No larvae exhibited detectable infections 7 days after treatment. At 14 days post-treatment, a significantly higher percentage of larvae were infected when pots received 1×10^7 spores than at any other dose. In the repeat of the drench experiment with extended incubation periods (14 and 21 days), there was also a significant interaction of day and dosage on the percentage of infected larvae ($F = 7.34$; df = 5, 70; $P < 0.0001$; Table 3). A significantly higher percentage of larvae were infected when pots received 1×10^7 spores after 14 and 21 days than at any other dose. The highest levels of infection were obtained in pots that received the highest spore dosage and were afforded an extended incubation period. All of the larvae in the drench studies were alive when collected from the pots. In these experiments, low effective dosages due to dilution of spores in the pots may have generated very low level infections that mimicked the effects seen at laboratory dosages of 10 spores per larva. Increased incubation periods

Table 3
Percentage of black vine weevil larvae infected from pots topically treated with various dosages of *Canningia* sp. spores

Spore dosage[a]	Experiment 1 Days post-application[b]		Experiment 2 Days post-application	
	7 Days	14 Days	14 Days	21 Days
0	0 ± 0	0 ± 0a	0 ± 0a	0 ± 0a
1×10^5	0 ± 0	0 ± 0a	—[c]	—
1×10^6	0 ± 0	1.7 ± 3.3a	0 ± 0a	2.0 ± 3.5a
1×10^7	0 ± 0	19.7 ± 11b	12.8 ± 10b	32.0 ± 14.5b

[a] Total number of spores applied to the surface of each pot.
[b] Means in the same column followed by the different letters are significantly different ($P < 0.05$; SAS Institute, 1999).
[c] Treatment not included in the second run of the experiment.

might well significantly increase detection of disease. Larval crowding can also serve as a stress factor that exacerbates microsporidian infection as reported for *Nosema pyrausta* (Paillot) (Microsporidia: Nosematidae) in *O. nubilalis* (Andreadis, 1986; Siegel et al., 1986). The overwintering mortality of insects infected with a microsporidium has also been shown to increase; for example, overwintering mortality of *O. nubilalis* larvae infected with *N. pyrausta* was significantly greater (+20%) than for uninfected larvae (Siegel et al., 1986; Kramer, 1959). *O. sulcatus* overwinters primarily in the final instar, often in high numbers in container-grown ornamentals, and under these conditions may suffer high levels of mortality due to *Canningia* sp. infection.

In these studies, we were unable to determine if *Canningia* sp. is vertically transmitted. Multiple attempts were made to infect late instar *O. sulcatus* with low dosages (10–100 spores) of *Canningia* sp. in order to determine its affect on adult longevity, fecundity and ability to be transmitted vertically. However, even ultimate instars inoculated with these very low dosages did not successfully pupate and eclose. We therefore infected preovipositional adults and were able to evaluate the impact of *Canningia* sp. infection on adult survival and fecundity, but were not able to determine if vertical transmission took place. Ingestion of *Canningia* sp. spores resulted in a significant reduction in adult survival ($F = 20.96$; df = 4, 137; $P < 0.0001$; Table 2). Strawberry 'Totem' leaves were fed to treated and control *O. sulcatus* adults based on reports that adults fed on this host plant species and variety have longer survival, a shorter preovipositional period (34 days), and higher fecundity (an average of 880 eggs per female) than adults fed on other common host plant species (Fisher, 2006). Adults in this experiment were afforded 42 days from spore feeding before being sacrificed to evaluate their infection status. Adults were allowed to oviposit for 3 weeks and control insects, none of which were positive for *Canningia* sp., laid an average of 30 ± 10 viable eggs per adult with a mean hatch of 70 ± 8%. A large proportion of adult *O. sulcatus* fed *Canningia* sp. spores became infected (20/27, 31/35, 20/22 and 24/24 of adults consuming 10^3 10^4, 10^5 and 10^6 spores, respectively, were infected). The only significant difference between the proportions of adults infected was a significantly higher proportion infected when fed 10^6 spores compared to those fed 10^3 spores. No adults infected with *Canningia* sp. oviposited, suggesting that vertical transmission is unimportant. *Canningia* sp. was observed in both larvae and adults in our prevalence survey and was originally isolated from an adult *O. sulcatus* collected from a wholesale nursery in McMinnville, OR. We speculate that because we were unable to observe completed development in any infected larvae, infected adults collected from the field probably acquired their infections as adults. The most likely source of inoculum would be in the soil from which the adults emerge. *O. sulcatus* pupate and eclose in the soil (Smith, 1932) and spores of *Canningia* sp. are shed in the feces (unpublished data). As eclosing adults emerge from the soil they may incidentally ingest spores left in the soil from feces and decomposing cadavers. The role of these adults in the epizootiology of the disease is currently unclear, particularly if infected adults are not able to oviposit. Nevertheless, spores may be shed in the feces of adults as well as larvae.

Canningia sp. was observed in all host collections with the exception of those received from New York State (Table 1). The absence of infected individuals from New York may have been an artifact of the relatively small sample size and a naturally low prevalence of the disease. Microsporidia typically produce chronic infections in the host (Solter and Becnel, 2007); individual hosts may survive for long periods of time and some microsporidian species are both vertically transmitted via infected females and horizontally transmitted, resulting in field prevalence that may reach relatively high levels (Lipa and Madziara-Borusiewicz, 1976; Wilson, 1977; Bruck and Lewis, 1999; Lewis et al., 2006). Conversely, insects infected with virulent pathogens that result in high early mortality may disappear from the host population quickly, resulting in low apparent prevalence due to difficulty in collecting living infected insects. The prevalence of the relatively virulent *Canningia* sp. in all samples was low, similar to other virulent microsporidia that are typically enzootic in the host population at low levels and rarely become epizootic, for example *Vairimorpha disparis* in *Lymantria dispar* (Pilarska et al., 1998).

Otiorhynchus sulcatus adults infected with as few as 1000 spores are completely unable to lay eggs. Vertical transmission, therefore, may be absent in adult hosts infected with *Canningia* sp. Pathogens of gut tissues, however, are typically effectively transmitted via the feces and individuals exposed as larvae in the laboratory with as few as 10 spores developed infections, suggesting that horizontal transmission of *Canningia* sp. in the field may be highly successful. Because *Canningia* sp. is an obligate pathogen, it is unlikely that it has a future as a traditional microbial insecticide. However, it may prove practical to apply *Canningia* sp. in an inoculative or augmentative approach in nursery or small fruit plantings to suppress populations of *O. sulcatus.* In potted plants, the microsporidium has potential to cycle, continuing to inoculate the potting media with spores and producing high mortality in such confined populations.

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